Title:

The role of β-defensin 126 on the ability of bull sperm to bind oviductal epithelium

Name: Alan Lyons

I.D: 11111704

Degree: MSc (Research) Animal Science

Supervisor: Dr. Seán Fair

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Declaration

I hereby declare that this project is my own original work and no part of it, except where fully referenced, is the work of anyone else.

Signed:  ____________________________

Date:    ____________________________
Abstract

Previous studies in primates have shown that β-defensin 126 regulates the ability of sperm to bind to oviductal epithelial cells, whilst in humans; mutations in the β-defensin 126 gene have been linked to male subfertility. Bovine β-defensin 126 (BBD126) exhibits preferential expression for the caudal epididymis of the bull but there have been no studies on its functional role in cattle. The aim of this study was to examine the role of BBD126 and BBD126 genetic variation on sperm motility, bovine oviductal epithelial cell (BOEC) binding ability and on sperm agglutination. In a previous study carried out by a member of our research group, Dr Emma Finlay, adjusted bull fertility phenotypes (based on a minimum of 1000 inseminations) for 7000 AI bulls were used to identify bulls of high and low fertility. The most divergent bulls (n=150) were selected for targeted sequencing of β-defensin genes and an association study was performed to identify genetic regions associated with sire conception rate. The most significantly associated single nucleotide polymorphisms (SNPs) were located in a haplotype consisting of 94 SNPs over 138kb, which included the β-defensin 126 gene, found only in bulls of high fertility. In this study, to examine the effect of the haplotype on sperm function, frozen-thawed sperm from high fertility bulls with (H+ive; n=4) and without (H-ive; n=4) the haplotype as well low fertility bulls without the haplotype (L-ive; n=4) were assessed for post-thaw motility using computer aided sperm analysis (CASA) and assessed for binding ability using in vitro BOEC binding assays (both monolayers and explants). BBD126 haplotype was found to have no effect on post-thaw sperm motility, however, bulls of high fertility with the haplotype (H+ive) had an increased ability to bind BOEC explants in comparison to bulls of high (H-ive) and low fertility (L-ive) without the haplotype (P<0.05). BBD126 has been shown to be highly resistant to methods of dissociation used in other species and, as a result, corpus epididymis sperm, a model in which the protein is not present, was used to study the functional role of BBD126 in sperm binding and agglutination. Corpus sperm were incubated with
recombinant BBD126 (rBBD126) in the absence or presence of BBD126 antibody. Addition of rBBD126 enhanced the ability of sperm to bind BOEC and reduced sperm agglutination (P<0.05). The presence of the antibody inhibited the increase in sperm binding ability, however, it failed to abrogate the effect of the protein on sperm agglutination. These findings indicate that BBD126 and BBD126 haplotype plays a role in bovine sperm binding and that BBD126 protein has a non-specific effect on sperm agglutination.
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List of Abbreviations

AI Artificial Insemination
ANAXA1 Annexing Protein 1
ANAXA2 Annexing Protein 2
ANAXA4 Annexing Protein 4
ANAXA5 Annexing Protein 5
BBD126 Bovine β-defensin 126
BBD126 Ab Bovine β-defensin 126 Antibody
BCF Beat Cross Frequency
BinB Rat β-defensin 1
BOEC Bovine Oviductal Epithelial Cells
BSP1 Binder of Sperm 1
BSP3 Binder of Sperm 3
BSP5 Binder of Sperm 5
Ca$^{2+}$ Calcium Ion
cAMP  Cyclic Adenosine Monophosphate
CASA  Computer Assisted Sperm Analysis
Ca   Cauda Epididymis
Co   Corpus Epididymis
Defb126  β-defensin 126
Defb1  Mouse β-defensin 1
Defb15  Mouse β-defensin 15
Defb16  Mouse β-defensin 16
Defb22  Mouse β-defensin 22
dH₂O   Distilled Water
DNA  Deoxyribonucleic Acid
EBI   Economic Breeding Index
ECM  Extracellular Matrix
HA   Hyaluronic Acid
hBD5  Human β-defensin 5
hBD6  Human β-defensin 6
HBP  Heparin Binding Protein
ICBF  Irish Cattle Breeding Federation
IgG  Isotype Control Immunoglobulin
NCBC  National Cattle Breeding Centre
NRR  Non-return Rate
PBS  Phosphate Buffered Saline
PR  Pregnancy Rate
rBBD126  Recombinant Bovine β-defensin 126
ROS  Reactive Oxygen Species
RT  Rete Testis
s.e.m.  Standard error of the mean
SNP  Single Nucleotide Polymorphism
ST  Seminal Tubule
STR  Straightness
UTJ  Utero-tubule Junction
VAP  Average Path Velocity
VD  Vas deferens
VCL  Curvilinear Velocity
VSL  Straight Line Velocity
Chapter 1: Introduction
Chapter 1: Introduction

1.1 The Irish Dairy Industry and Fertility

Ireland, like New Zealand, operates a seasonal grass-based system. This production system possesses a significant cost advantage in the form of low-cost milk production as the calving season is synchronised with the availability of low-cost grass (Berry, 2015). As a result, high reproductive performance is essential to ensure as many cows as possible calve at the start of the grass-growing season (Dillon et al., 1995). Whilst milk production has more than doubled per cow since the 1970’s, there was a dramatic decline in the reproductive performance of high-yielding dairy cows in the same period (Walsh et al., 2011). Up until the 2000’s, the additional revenue generated from the increase in milk production was eroded by the concurrent decline in fertility—primarily a result of early embryo loss (Diskin and Morris, 2008; Berry, 2015; Figure 1).

On Irish dairy farms, the target is for each cow to produce a calf every 365 days, however, in 2013 the average calving interval was 394 days, with a six-week calving rate of 58% (ICBF, 2013). The six-week calving rate is the percentage of cows that had calved within the first six weeks of the calving season (Macmillan, 2012). It has been estimated that for every 1% increase in the six-week calving rate, farm profitability increases by €9.26 per cow per year and €3.51 per heifer per year (Shalloo et al., 2014).

This reduction in dairy cow fertility was known to have a significant genetic component (Minten et al., 2013). In 2001, an Economic Breeding Index (EBI) was introduced in Ireland to improve the genetic merit of the national herd (Figure 1). The EBI is a single-figure profit index devised to help farmers identify the most profitable bulls and cows for breeding dairy herd replacements (Berry et al., 2005). Currently, it comprises of six sub-indices – the two most important being the milk production and fertility sub-indices (Ramsbottom et al.,
Presently, the dairy industry has entered the genomics era, the selection of breeding bulls is now based on genomic selection markers associated with milk production, body conformation, longevity and fertility (Hung and Suarez, 2010, Spelman et al., 2013; Figure 1). Genomics has increased the reliability of the EBI, doubled the rate of genetic progress and facilitated a shortened generational interval (Berry, 2015). The generational interval – the average age of parents at birth of their offspring, has shortened as animals can now be selected for breeding at a younger age. This is helping to reverse the trend of declining reproductive performance of lactating Holstein cattle (Amann and DeJarnette, 2012; Figure 1). However, genomics has also led to the problem that young bulls that are in high demand are used extensively once they can produce sufficient quantities of semen. As a result, there is insufficient time to test inseminations in the field, therefore field fertility is unknown (Amann and DeJarnette, 2012).

Figure 1: The impact of the EBI and genomics on Irish dairy farm profitability over time (Kearney, 2016).
1.2 Male Fertility

A significant percentage of reproductive difficulty in cattle can also be ascribed to bull subfertility (DeJarnette et al., 2004). Pregnancy rates are 50-55% in Irish dairy cows but can fall as low as 25% due to variation in the bull used (Murphy et al., 2015, Al Naib et al., 2011). The primary goal of dairy producers is to achieve a high pregnancy rate (PR) with semen from genetically superior bulls (Amann and DeJarnette, 2012). Currently, there is no single diagnostic test to accurately predict fertility in bulls producing apparently normal semen (Al Naib et al., 2011). Common semen quality measures such as sperm concentration and sperm motility which are used to characterise male reproduction often do not correlate with fertility rates in the field (Parkinson, 2004). This variation in bull conception rate is a major impediment to the use of high genetic merit bulls, particularly as young genomically selected bulls are used extensively without testing their in vivo fertility. There needs to be an increased emphasis on estimated fertility when selecting a bull (Miglior et al., 2005). Presently, the majority of genomic research is predominantly focused on female phenotypes and, despite their important role in reproductive success; variation in male traits has been poorly investigated (Capitan et al., 2015).

β-defensins are a family of peptides of innate immune response - known as host defense-peptides, recently discovered in bovines (Cormican et al., 2008). As well as their antimicrobial function they are now known to have immunomodulatory function and have been shown to play a role in fertility in primates and rodents (Tollner et al., 2008a, Tollner et al., 2011, Yudin et al., 2008). Despite the evidence linking β-defensins and fertility in other species (Dorin and Barratt, 2014), almost nothing is known about the functional role of these genes in cattle. Through increased understanding of bull sperm biology and specifically the role of β-defensins it may be possible to devise novel tests to help improve bull fertility and artificial insemination (AI) practices.
1.3 Male Reproductive System

The male reproductive anatomy consists of the testis, the spermatic cord, the epididymis, accessory sex glands and the penis (Senger, 2005). The testes consist of 200-400 compartments divided by fibrous connective tissue called lobules (Senger, 2005). The epididymis then provides the environment in which sperm matures and serves as a storage facility for sperm (Senger, 2005; Figure 2).

![Reproductive anatomy of the bull](senger2005.png)

**Figure 2:** Reproductive anatomy of the bull (Senger, 2005).

1.3.1 Epididymal Sperm Maturation

Spermatogenesis provides the male with a continuous supply of genetically diverse gametes and has been identified as one of the major sources of genetic diversity within a species (Sofikitis et al., 2008, Ellegren, 2007, Johnson et al., 2000). However, this process produces a non-functional male gamete that has no transcriptional or translational abilities due to a
highly condensed chromatin (Grunewald et al., 2005). Prior to successful fertilisation, the sperm must undergo maturation in the epididymis including the acquisition of motility and fertilising ability (Caballero et al., 2011). The epididymis can be divided into three sections; the caput epididymis (head), corpus epididymis (body) and the caudal epididymis (tail); (Figure 3).

**Figure 3:** Bull testis detailing the epididymal sections including caput, corpus and cauda (Senger, 2005)
Maturation entails a number of bio-chemical modifications to increase the functional integrity of the sperm (Cornwall, 2009). The interaction of steroid hormones, testicular factors, and other elements such as temperature, pH, and osmotic pressure creates a unique physiological environment in each of the epididymal sections (Robaire and Viger, 1995). Sperm progress along the duct as the smooth muscle contracts, with the muscle layer increasing progressively as the sperm move from the caput to the caudal epididymis. In the bull, this epididymal transit takes 7-10 days (Hafez and Hafez, 2000).

1.3.1.1 Caput Epididymis

The caput is responsible for the early stages of maturation of the sperm. The caput reabsorbs fluid from the rete testes via the efferent ducts (Cornwall, 2009). Immotile sperm enter the cauda and remain immotile until they reach the corpus. This is primarily due to the low concentrations of cyclic adenosine monophosphate (cAMP) present which inhibits movement of the sperm tail (Guyonnet et al., 2009).

1.3.1.2 Corpus Epididymis

Concentrations of cAMP increase in the corpus in response to signalling by the sperm plasma membrane and bind to its subunit cAMP dependent protein kinase A (Pariset et al., 1985). This increase in the cAMP concentration leads to the development of a chaotic trashing type movement of the sperm tail. Furthermore, forward motility protein (FMP) is secreted by the epithelium of the corpus and leads to a subsequent increase in forward motility in the cauda (Ding et al., 2007). FMP converts the initial chaotic movement of the tail to forward progressive movement (Acott et al., 1983).
1.3.1.3 Cauda Epididymis

Transit through the epididymis evokes a restructuring of membrane proteins and lipids as part of the maturation process. The cauda secretes proteins, adhesion molecules and ubiquitin, which are involved in the elimination of defective sperm. Proteins secreted by the cauda include: enzymes for modification of proteins and membrane surface lipids (Girouard et al., 2011) and proteins involved in sperm fertilising ability (Netzel-Arnett et al., 2009) and membrane protection (Nagdas et al., 2014). Studies carried out in bovine demonstrated that serine and cysteine protease inhibitors secreted in the cauda protect membrane-sperm from inappropriate proteolytic degradation (Reyes-Moreno et al., 2002). Furthermore, concentration of P25b - a protein involved in the acquisition zona pellucida binding ability, increases from the corpus to the caudal epididymal regions (Caballero et al., 2011).

1.4. β-defensins

1.4.1. Evolution of β-defensins

Androgens – male sex hormones including testosterone and dihyrdotestosterone are the primary modulators of gene expression in the epididymis (Ribeiro et al., 2016). Among these genes, several encode for the β-defensin gene family, which have a wide taxonomic distribution. They are found in vertebrates, invertebrates and also in plants (Meade et al., 2014). The β-defensin gene family exhibits species specific variation in β-defensin gene number. Currently, defensin gene number varies from 14 genes in chickens, to 29 in pigs, 33 in chimps, 48 in humans and 57 in bovines (Cormican et al., 2008, Choi et al., 2012, Radhakrishnan et al., 2007, Meade et al., 2014, Patil et al., 2005). This divergence in gene number is thought to be due to genetic drift and variation in selective pressures. β-defensin genes exist in a single cluster in birds, whereas, they exist as four gene clusters in dogs, rats, mice, and cattle (Meade et al., 2014).
A unique cluster of 19 β-defensin genes in bovines has been characterized (Meade et al., 2014). This newly discovered cluster spans 320 kb on chromosome 13 (Cormican et al., 2008). Sixteen of these genes share an orthologous relationship with humans and canines (Figure 4). However, the bovine genome appears to encode a number of relatively recent gene duplicates within this cluster. As a result, cattle have the most diverse repertoire of genes so far identified (Meade et al., 2014). Strong evolutionary pressures in this lineage have selected for the development of enlarged sets of multifunctional β-defensins (Meade et al., 2014). In mice, humans and macaques, key roles are emerging for these genes in reproduction (Tollner et al., 2008a, Yudin et al., 2008, Tollner et al., 2008b) and immunity (Funderburg et al., 2007). As a result, genetic variations i.e. polymorphisms within these genes hold exciting potential for improving bovine fertility and breeding practices (Meade et al., 2014).

1.4.2. β-defensin Structure

Most β-defensin genes have a characteristic two exon structure, the first of which encodes a prepropeptide with a hydrophobic leucine-rich signal sequence, while the mature peptide is encoded by the second exon (Meade et al., 2014) As a family, β-defensins can be characterised by their cationic nature and high sequence variability in the mature peptide, with only the signal peptide and 6 cysteines showing high sequence conservation (Meade et al., 2014, Narciandi et al., 2011). The defensin motif, conserved across peptides and species, is formed by three disulfide bonds in an orderly manner (1–5, 2–4, 3–6), thus stabilising the correct folding of the peptide (Narciandi et al., 2011). The specific C1-C5, C2-C4, and C3-C6 cysteine pairing, which is conserved across all β-defensins, indicates that the disulfide bonds are essential to the function of the molecule.
**Figure 4:** Syntenic map of bovine, human, and canine β-defensin genes in a direct 1:1 relationship mapping to Bovine chromosome 13 cluster. Bovine in blue, Human in green, and Canine in red (Meade et al., 2014).
1.4.3. β-defensins Function

In the past, β-defensins were thought to be pore-forming peptides that aggregated on the surface of bacterial cells to cause cell leakage and subsequent cell death (Selsted and Ouellette, 2005). However, recent studies have revealed multiple complex mechanisms by which these proteins mediate numerous other biological activities, including immune regulation (Semple and Dorin, 2012) and fertility (Tollner et al., 2008a, Tollner et al., 2008b). β-defensins play a role in fertility in a number of species including humans (Hollox et al., 2008), mice (Yudin et al., 2008, Zhou et al., 2004) and other primates (Tollner et al., 2012).

The microcidal properties of the β-defensin family are well documented in mice and humans (Semple et al., 2015). In humans, β-defensin 3 has been found to disrupt staphylococcal cell wall biosynthesis (Sass et al., 2010). In mice, β-defensin 10 has been shown to have antimicrobial effects in vivo (Peyrin-Biroulet et al., 2010). In addition to displaying potent microcidal properties, β-defensins have a significant role in other aspects of innate and adaptive immunity and have been associated with the development of pro-inflammatory biological responses (Meade et al., 2014). These effects occur as a result of β-defensins’ ability to bind to a variety of ligand receptors resulting from their cationic and promiscuous nature (Semple and Dorin, 2012).

In the male, multiple β-defensins act in a synergistic and sequential manner in the epididymal luminal fluid, contributing collaboratively in this way to enable sperm protection, maturation and to gain fertilisation ability along the epididymal tract (Semple and Dorin, 2012). The first defensin-like peptide to be isolated from the epididymis was Bin1b in rats (Semple and Dorin, 2012). Bin1b was shown to be present in the main cluster of β-defensin genes. The Bin1b structure exhibits a positive charge and conserved cysteines, which correspond with the β-defensin family. Bin1b has been shown to be exclusively...
expressed in the epididymis of the rat binds to the sperm surface and induces Ca\(^{2+}\) uptake resulting in sperm becoming progressively motile (Zhou et al., 2004).

In humans, \textit{hBD5} and \textit{hBD6} (\textit{β-defensin 5} and 6) are expressed in the epididymis, particularly in the caput region (Zaballos et al., 2004). Orthologs of these genes (\textit{Defb12} and \textit{Defb15}) are also expressed in mice (Yamaguchi et al., 2002). These epididymally expressed defensins exhibit antimicrobial properties (Yamaguchi et al., 2002). Lipopolysaccharide induced inflammation of the epididymis decreased expression of β-defensins in the caput (Cao et al., 2010). However, most notably, this reduction in defensins also reduced sperm motility. This finding is further substantiated by studies of β-defensins in other species. In rats, a knock down study of \textit{Defb15} led to a decline in both total and progressive motility (Zhao et al., 2011). In humans, lower levels of \textit{Defb1} are associated with reduced motility as well as lower bactericidal activity (Diao et al., 2014).

\subsection{1.4.4. \textit{β-defensin 126} and Fertility}

β-defensins and specifically β-defensin 126 (\textit{Defb126}) have been found to play a significant role in sperm maturation and fertility (Zhou et al., 2004). In the macaque, \textit{Defb126} is secreted in the corpus and cauda epididymis where it has been reported to bind to the entire sperm surface (Yudin et al., 2005). This coating protein contains multiple sialylated oligosaccharides which enhance migration of sperm through cervical mucus \textit{in vitro} (Tollner et al., 2008b). By increasing the negative charge on the sperm, \textit{Defb126} enables progression through the electro-negative mucus more efficiently (Tollner et al., 2008a). Furthermore in the macaque, \textit{Defb126} is known to play an integral role in sperm binding to oviductal epithelial cells (Yudin et al., 2005, Tollner et al., 2008a).

Additionally, there is now considerable evidence indicating a role of orthologs of the β-defensin 126 gene in fertility regulation in humans (Tollner et al., 2011). A polymorphism
found in the human β-defensin 126 gene has been found to correlate with reduced fertility (Tollner et al., 2011). In humans, a recently discovered variant in Defb126 that has a 2-nucleotide deletion in the open reading frame, which generates a non-stop mRNA, has been linked with reduced male fertility (Tollner et al., 2011). Men who are homozygous for the mutation produce sperm that have a deficit in O-linked oligosaccharides on the surface of the sperm head and exhibit an 84% reduction in the rate of penetration of a hyaluronic acid gel (HA), a surrogate for cervical mucus, occurred in comparison with the other β-defensin 126 genotypes (Tollner et al., 2011). Most notably, with respect to common measures of semen quality parameters e.g. sperm progressive motility and sperm morphology, these men appear normal and resemble men with the Defb126 allele. However, using genotypic analysis of a population-based cohort found that men with the homozygous genotype are significantly less fertile ~ 60%, than men with the Defb126 allele (Tollner et. al, 2011).

1.4.5. Bovine β-defensin 126

Bovine β-defensin 126 (BBD126), the ortholog of human Defb126, has been shown to have localised site-specific expression in the caudal epididymis exclusively in the reproductive tract of the mature bull (Narciandi et al., 2011; Table 1). The Class A group of genes (BBD132, 129, 128, 127, 126, 125 and 125a) were shown to be expressed in the mature bull epididymis and vas deferens, but were completely absent from the immature male sampled and the female samples suggesting that they may be developmentally regulated and also suggests a potential role in sperm maturation for these genes (Narciandi et al., 2011) Expression analysis of β-defensins in the genital tract of other species showed quantitative variation in gene expression in different sections of the reproductive tract, suggesting that site-specificity of gene expression may reflect differences in the biological role of these defensins (Zhou et al., 2004, Narciandi et al., 2011).
The Class A genes possess additional characteristics other than expression pattern which differentiates them from the other genes in the cluster such as significantly extended C-termini compared with the rest of the genes in the cluster. Class A genes have a tail of fifty amino acids on average, which is significantly longer than the twenty-four amino acids of other genes in the cluster, increasing the chances for glycosylation of the peptide (Narciandi et al., 2011). In humans, experimental removal of these glycan structures reduces the peptide in size from $>32$ kDa to $<10$ kDa illustrating the extent to which Defb126, in particular, is glycosylated (Tollner et al., 2012). However, cleavage of surface carbohydrate residues from BBD126 did not change the size of the peptide detected using Western-blotting (Narciandi et al., 2016). The glycosylation patterns of human and bovine orthologs have been shown to be dissimilar as a result of differences in the C-terminus of the respective peptide sequences, where the glycosylation motifs are predicted to occur. Defb126 has been shown to have 52 amino acids in its C-terminus, whereas BBD126 has 30 (Narciandi et al., 2016).
Table 1: Summary representation of patterns of expression detected in 6 mature (18 months old) bulls, a 43 days old immature bull and 10 cows for each gene compared with the control gene (GAPDH) across reproductive tracts. −, indicates no detectable gene expression; +, gene expression 10⁻⁴ fold lower than GAPDH; ++, gene expression between 10⁻⁴ and 10⁻² fold change lower than GAPDH; ++++, gene expression between 10⁻² and 1 fold change of GAPDH; ++++, gene expression higher than GAPDH. ST, seminal tubule; RT, rete testis; Ca, caput; Co, corpus; Cu, cauda; VD, vas deferens; T, testis; Ov, ovary; PFT, proximal oviduct (isthmus); DFT, distal oviduct (ampulla) and U, uterus (Narciandi et al., 2011)

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Furthermore, *in silico* analysis identified 9 and 14 predicted O-linked glycosylation sites in the human and murine 126 peptide sequences, respectively. However, in the orthologous bovine sequence, only one site was predicted, therefore, indicating limited evidence for glycosylation of BBD126, potentially highlighting a species-specific difference between these orthologous genes (Narciandi et al., 2016).

Extensive staining of BBD126 has been shown on bull sperm, with higher localisation predominantly on the tail and dorsal section of the head (Figure 5) and in suspension on the seminal plasma (Fernandez-Fuertes et al., 2016) Interestingly, the lack of BBD126 peptide expression in seminal plasma collected from vasectomised bulls rules out the expression or secretion of BBD126 from any accessory sex glands in the bull (Narciandi et al., 2016, Fernandez-Fuertes et al., 2016).

**Figure 5:** Caudal bull sperm labelled with anti BBD126 (Fernandez-Fuertes et. al., 2016).
The binding pattern of BBD126 (Fernandez-Fuertes et al., 2016) has been shown to differ from that described for the macaque ortholog, which coats the entire sperm surface (Yudin et al., 2003) and the mouse ortholog, which is present over the entire sperm surface except for the equatorial segment (Yudin et al., 2008)

Another difference between the bovine and macaque orthologs seems to be the method by which these proteins attach to the sperm surface. GPI-anchored proteins are a major component of the lipid rafts in sperm and are involved in the membrane reorganization that takes place during the capacitation of the male gamete in the female reproductive tract (Boerke et al., 2014). Bovine sperm treated with PIPLC, an enzyme that cleaves off GPI-anchored proteins, retain BBD126 in their structure (Fernandez-Fuertes et al., 2016), whereas in macaque sperm it is removed (Yudin et al., 2003), indicating that BBD126 attaches to the sperm surface through a different mechanism than in the macaque.

A previous study in the macaque, demonstrated that a combination of caffeine and dbcAMP synchronizes sperm capacitation in this species and, as a result, removes Defb126 from their surface (Tollner et al., 2004). However, incubation of bovine sperm with the same capacitation inducing treatments failed to remove BBD126 (Fernandez-Fuertes et al., 2016). Increasing the duration of incubation, the concentration of the different reagents or incubating sperm overnight prior to exposing them to the treatments did not result in a loss of the protein (Fernandez-Fuertes et al., 2016). This study suggests that bovine BBD126 is retained after sperm undergo this process, in contrast to the macaque (Tollner et al., 2004). Similar results have been observed for the mouse ortholog, where sperm recovered from both the oviduct and from the surface of cumulus-oocyte complexes showed immunorecognition of Defb22 over their entire surface (Yudin et al., 2008). However, sperm found within the cumulus cell matrix of the oocyte had lost most of the Immunofluorescence over the sperm head, while retaining it along the tail and midpiece (Yudin et al., 2008).
suggests that in the mouse there is a region-specific loss of Defb22, probably induced by the acrosome reaction, which might also be the case in bovine sperm (Fernandez-Fuertes et al., 2016).

BBD126 has been shown to exist as a primer-dimer which is highly resistant to standard methods of dissociation (Narciandi et al., 2016). The formation of β-defensin dimers has been described in other species; Defb126 is thought to interact with the lipid membrane on sperm as a covalently linked dimer, whereas Defb22 in rats also exists as a disulphide linked homodimer (Campopiano et al., 2004, Song et al., 2011, Zanich et al., 2003). In one study, it was found only a prolonged incubation at 95°C could completely disrupt the dimer into BBD126 monomers of the expected size (Narciandi et al., 2016), whereas Defb126 in macaques, has been reported to be readily lost during the process of capacitation (Tollner et al., 2004). In humans, Defb126 tightly adheres to the sperm surface, resisting removal by centrifugation through density-gradient solutions and high salt conditions (Tollner et al., 2004). The phenomenon of dimerisation in these peptides with highly conserved cysteines has been described as a disulphide-lock (Wommack et al., 2014). Interestingly, BBD126 has an extra (7th) cysteine, potentially creating an even more powerful disulphide-lock (Narciandi et al., 2016).
1.5. β-defensins and Sperm Transport in the Oviduct

Sperm transport can be split into two phases: rapid and sustained transport. Population of the oviduct by some sperm occurs moments post copulation, this rapid transport, is the result of an initial burst of energy trigged post ejaculation and by the contraction of the female reproductive tract. However, these sperm are unlikely to be able to fertilise the oocyte as sperm must reside within the female tract for a period of time prior to fertilisation (Knobil and Neill, 2006). Sperm are required to undergo morphological changes i.e. capacitation before they are able to successfully fertilise an ovum (Marquez and Suarez, 2007). Capacitation is the programmed maturation of sperm as they undergo a number of biochemical modifications that exposes zona pellucida binding proteins that are required for fertilisation (Miller, 2015). This process involves an increase in membrane fluidity, subsequent influx of Ca\(^{2+}\), and an increase in cAMP levels (Parrish, 2014). Sustained transport involves a trickle of viable and motile sperm cells released into the ampulla of the oviduct – the site of fertilisation in the cow – over a sustained period of time. These are released from a sperm binding reservoir formed in the isthmus of the oviduct (Knobil and Neill, 2006).

The bovine oviduct is a pair wise tubular structure, about 20 to 28 cm in length, embedded in a serosa fold – the mesosalpinx (Rottmayer et al., 2006, Coy et al., 2012). The oviduct provides a connection between the ovary and uterine horn. It consists of five morphological and functional distinguishable regions – utero-tubal junction (UTJ), isthmus, ampullary-isthmic junction, ampulla and infundibulum (Figure 6).
1.5.1. The UTJ

In cows, sperm are deposited in the anterior vagina post coitus (Coy et al., 2012). Sperm must then cross the cervix and navigate through the uterus. However, in AI, sperm are deposited in the body of the uterus and bypass the necessity of this sperm transport mechanism (Seidel and Schenk, 2008); Figure 7).

Figure 6: Anatomical sections of the bovine oviduct. 1. Utero-tubal junction (UTJ) 2. Isthmus 3. Ampullary-isthmic junction 4. Ampulla 5. Infundibulum

Figure 7: Artificial insemination (AI) in cattle is performed intra-uterine (transcervical) with sperm placement occurring in the body of the uterus via catheter.
Once in the uterus, sperm must pass the UTJ to reach the isthmus and ampulla of the oviduct (Miller, 2015). The UTJ marks the distal end of the oviduct (Hung and Suarez, 2010). In cows, the micro-architecture of the UTJ consists of folds in the lining of the junction. These folds form cul de sacs with the open end facing the uterus (Yaniz et al., 2000); (Figure 8). This micro-architecture of the UTJ itself may play a role in sperm selection as a very low percentage of the sperm population reach the oviduct past the UTJ (Coy et al., 2012). When the lumen of the oviduct is compressed due to muscular and/or vascular action, the cul de sacs found in the folds can form a plug (Hung and Suarez, 2010). This mechanism may help regulate the numbers of sperm that reach the ampulla, thereby reducing the incidence of polyspermic fertilisation (Hunter and Wilmut, 1984). On the other hand, when the UTJ is not being compressed, the dead ends of the channels can act like funnels to direct sperm into the site of sperm binding – the isthmus (Hung and Suarez, 2010).

**Figure 8:** Surface morphology of the utero-tubal junction, opened longitudinally, and detailing the arrangement of secondary folds forming cul-de-sacs with their opening pointing to the uterus, SEM X15 (Yaniz et al., 2000).
For successful UTJ passage, sperm are required to be progressively motile and to express specific proteins (Hung and Suarez, 2010). In mice, this is evidenced by the absence of sperm deficient in the specific protein ADAM3 beyond the UTJ (Okabe, 2013).

1.5.2. The Isthmus and Sperm Binding Reservoir Formation

The location of sperm storage varies between species (Miller, 2015). This variation may be related to the morphological differences in the female reproductive anatomy (Neubaum and Wolfner, 1999). Due to the species specific variation in sperm storage mechanisms, this ability has likely appeared independently and relatively recently (Holt and Lloyd, 2010). In bovines, the convoluted isthmus with its narrow lumen acts as the functional sperm reservoir (Suarez et al., 1997). In comparison to the ampulla, the isthmus lumen is particularly tortuous due to mucosal folding and flexure resulting in increased numbers of sperm binding sites (Yaniz et al., 2000). Millions of sperm must be artificially inseminated in order to fertilize only one oocyte (Foote and Kaproth, 1997). Of the millions initially inseminated, only thousands pass the UTJ and reach the isthmus of the oviduct (Suarez, 2016). Most of the sperm that pass into the oviduct bind to the oviductal epithelium (Figure 9). However, in vitro, sperm have been shown to fertilise oocytes without binding to epithelial cells.
This reservoir plays a critical role in successful fertilisation *in vivo* as sperm binding extends viability. The head of sperm binds to epithelial cells lining the oviduct (Suarez and Pacey, 2006). This epithelial cell layer consists mainly of two different cell types, ciliated and secretory cells (Figure 10). Cilia are microtubule-based organelles located on the surface of epithelial cells, ranging from 5-10 μm in length and 300 nm in diameter, which beat periodically (Rottmayer et al., 2006). Secretory cells possess numerous microvilli on their apical side which are made of actin filaments which are 1-2 μm in length (Rottmayer et al., 2006). Secretory cells secrete oviductal specific glycoprotein which has been shown to have a role in the regulation of zona pellucida sperm binding (Coy et. al., 2008).

*Figure 9:* Bull sperm attached to bovine oviductal epithelium post infusion in vivo, SEM X1700 (Lefebvre et al., 1995)
Sperm bind to the epithelium via the plasma membrane overlying the sperm acrosome (Suarez, 2002). Most often this binding occurs on ciliated cells (Lefebvre et al., 1995). Interestingly, however, scanning electron micrographs of oviducts taken from mated animals show sperm also bind with microvilli of non-ciliated cells (Hunter et al., 1991). Sperm attachment to microvilli is lower in avidity compared with cilium attachment, but may be enabled in vivo by grooves formed by secondary epithelial folding (Suarez and Pacey, 2006). This binding process is thought to activate production of enzymes that regulate reactive oxygen species (ROS) in sperm, which results in enhanced fertile sperm lifespan (Kawakami et al., 2001). The ability to maintain sperm fertile lifespan is not a common property of all epithelial cells, only oviductal epithelial cells (Boilard et al., 2002).

In addition to prolonging sperm viability, sperm storage sites recognize and select a fertile sperm population (Teijeiro et al., 2011). Epithelial cell binding appears to select for higher-

Figure 10: In the isthmus, non-protruding secretory cells are partially concealed by the cilia (a), except in the basement area of some pockets (Yaniz et al., 2000)
quality human sperm, as the population of attached sperm had significantly better motility, fewer membrane disruptions, and fewer abnormalities in chromatin structure than sperm that were not attached (Ellington et al., 1999). Capacitated sperm have a reduced ability to bind to epithelial cells in comparison to uncapacitated sperm (Machado et al., 2014, Tienthai et al., 2004). It is imperative capacitation occurs proximal to the ampulla to ensure successful fertilisation (Coy et al., 2012).

Sperm binding also regulates sperm function (Miller, 2015). While bound to oviduct epithelial cells, sperm are maintained in a protective state characterized by suppression of motility (Rodriguez-Martinez and Barth, 2007). Epithelial adhesion regulates sperm function by suppressing the normal increase in sperm intracellular free calcium (Dobrinski et al., 1997). The mechanism allows for successful fertilisation in species in which semen deposition and ovulation are not always synchronized as sperm have been shown to be released stochastically from the sperm reservoir during the oestrus cycle (Miller, 2015).

1.5.3. Bull Sperm Binding

1.5.3.1. Role of Glycans in Sperm Binding

There have been many studies of adhesive molecules that might be responsible for retaining sperm in the isthmus. However, the precise identity of the molecular interactions underpinning the establishment of the sperm reservoir remains unclear (Suarez, 2016). There is evidence in several species that sperm bind to the cilia via glycans found on oviduct epithelial cells (Lefebvre et al., 1997, Suarez, 2001, Wagner et al., 2002, DeMott et al., 1995).

In cattle, sperm binding is thought to be mediated by fucose recognition (Lefebvre et al., 1997). Fucose, found specifically in the Lewis A trisaccharide (Figure 11), when added to epithelial cells prior to sperm addition in vitro, has been found to have a significant inhibitory
effect on bull sperm binding (Suarez, 2016). This reduction in binding to oviduct cells supports the notion that Lewis A is related to the authentic oviduct glycan that binds sperm (Figure 11). However, the majority of studies have tested very high concentrations of just a few monosaccharides or small oligosaccharides. Thus, the definitive identity of the oviduct glycan that binds sperm remains uncertain (Suarez, 2016).

1.5.3.2 Role of Annexing Proteins & the Chaperones

It is proposed that oviduct plasma membrane annexin proteins containing Lewis A bind to surface proteins deposited on sperm at ejaculation (Ignotz et al., 2007). These annexin proteins (ANXA1, -2, -4, and -5) have been shown to interact with sperm surface proteins (Figure 11) and to be immunolocalized to the surface of oviductal epithelium. Antibodies to annexins have been shown to reduce sperm binding in vitro (Ignotz et al., 2007).

Conversely, a study by another group has indicated that two bovine oviductal proteins, the chaperones GRP78 and HSP60, also mediate sperm binding (Boilard et al., 2004). As a result, whilst both the annexin proteins and the chaperones have been tentatively identified as oviductal receptors, understanding of this biological receptor mechanism remains limited (Suarez, 2016). In some species, binding of sperm to these epithelial cell receptors appears to be mediated by lectin-like sperm binding proteins e.g. binder of sperm 1 (BSP1), that adhere to the surface of sperm (Tollner et al., 2008a; Figure 11)
1.5.3.3. Role of Sperm Binding Proteins

BSP1 is a product of bovine seminal vesicles (Salois et al., 1999), which is present in seminal plasma at concentrations of 15–50 mg/ml (Nauc and Manjunath, 2000). BSP1, formally known as PDC-109, coats the anterior head of ejaculated bull sperm but not epididymal sperm (Suarez, 2016). BSP1 binds to choline phospholipids via short hydrophobic sequences (Ramakrishnan et al., 2001). This characteristic is thought to underpin BSP1 adsorption onto sperm during and after ejaculation (Desnoyers and Manjunath, 1992). BSP1 appears to be lost from the plasma membrane overlying the sperm head during capacitation (Therien et al., 2001). This capacitation-associated loss of BSP1 results in reduced sperm binding to epithelial cells. This loss can be restored when sperm are removed from capacitating conditions and treated with BSP1 (Gwathmey et al., 2003).

Figure 11: Sperm binding mechanism in bovine illustrating the possible role of fucose, Lewis A, sperm binding proteins and annexing proteins.
Furthermore, two other BSP proteins, BSP3 and BSP5 have been found to enhance sperm binding to oviductal epithelium (Gwathmey et al., 2006). All three BSP proteins are produced by the seminal vesicles (Tollner et al., 2008a). However, the concentrations of BSP3 and BSP5 (2–6 mg/ml) are only about one-tenth the concentration of BSP1 (Nauc and Manjunath, 2000). Each BSP has been shown to enhance sperm binding to oviductal epithelium independently. Formations of heteromeric complexes are not required. A heteromeric complex requires a number of protein sub units to combine to enable the protein to function. As a direct consequence, since each BSP can act independently, each may play a different, if overlapping role in mediating sperm interactions with oviductal epithelium (Suarez, 2016; Figure 11).

In macaques, Defb126 is a sperm surface-coating protein and has a role in capacitation and fertilisation. Defb126 is a major component of the sperm surface glycocalyx (Yudin et al., 2005). The glycolax is a dense coating of carbohydrate-rich molecules and glycoproteins (Schroter et al., 1999). Defb126 is bound to the entire surface of ejaculated macaque sperm (Yudin et al., 2003) and it must be released from the sperm surface in order for sperm to bind to the zona pellucida (Tollner et al., 2004). Loss of Defb126 from the sperm head with capacitation is associated with a significant loss of the ability of sperm to bind to epithelial cells (Tollner et al., 2008a).

1.5.4 Bull Sperm Release from Epithelial Cells

In cattle, viable sperm are stored in the isthmic region of the oviduct for approximately 18-20 hours (Hunter and Wilmot, 1984). Prior to ovulation, which occurs 28-31 hours after the onset of oestrus in cattle, sperm are released to ascend into the ampulla (Hunter and Wilmot, 1984). The ampulla-isthmic junction is the point of convergence between the isthmus and the ampulla, and as result exhibits characteristics of both parts (Rottmayer et al., 2006). The ampulla, representing two thirds of the oviduct length, is the wider part of the tube, where
oocyte maturation and fertilisation take place. It is connected to the narrower section by the ampullary-isthmic junction (Rottmayer et al., 2006).

It is unlikely that the release of sperm is due to the loss of binding sites on the oviductal epithelium, as sperm have been shown to bind to epithelial cells from different stages of the oestrous cycle (Lefebvre et al., 1995, Gwathmey et al., 2003, Baillie et al., 1997). Rather, it is likely that hormonal changes i.e. elevated oestrogen or luteinising hormone, which trigger ovulation also stimulate the release of factors in the oviduct that cause changes in sperm which enable them to release themselves from the oviductal epithelium (Miller, 2015). There are several models being tested to explain sperm release. It is postulated that there is a controlled release of stored sperm near ovulation in response to chemotactic signals from the ovulated oocyte. The trigger for sperm release may be due to a biochemical alteration in the sperm or in the fluid surrounding the cells (Miller, 2015).

1.5.4.1 Capacitation

This initial increase in membrane fluidity resulting from capacitation increases cell membrane permeability. Subsequently, this enhanced permeability results in a Ca$^{2+}$ ion influx. This triggers a cascade which results in cell hyperactivity and increased sperm motility. Post capacitation, the cells switch from a linear rotation to a more frenzied movement (Breininger et al., 2010). In addition, capacitation also involves biochemical changes to the sperm head (Marquez and Suarez, 2007). Accessory glands of the male reproductive system secrete heparin binding proteins (HBP), which coats the seminal glycolax. Subsequently, as cells detach from the epithelial lining the glycolax is removed (Kumar et al., 2009; Figure 12)
1.5.4.2 Hyperactivation

For successful fertilisation to occur, the waveforms of the sperm flagellum must be altered dramatically as the sperm travels through the female reproductive tract (Simons et al., 2014). The most important change required is the transition from a symmetric, flagellar beat form to an asymmetric beat form with larger bending amplitudes along the flagellum (Simons et al., 2014, Singh and Rajender, 2015). This change is defined as hyperactivation (Figure 13).

Figure 12: Capacitation triggered sperm release from ciliated epithelial cells and seminal glycolax removal

Figure 13: Bull sperm swimming patterns. A) Activated sperm exhibiting symmetrical flagellar beating. B) Hyperactivated sperm displaying high amplitude and asymmetrical flagellar movement. C) Maximal form of hyperactivation, the figure-eight pattern (Simons et al., 2014).
Hyperactivation of sperm occurs as sperm reach the oviduct of the female reproductive and is an integral part of the capacitation process (Curtis et al., 2012, Ho and Suarez, 2003). It manifests as a rapid movement of sperm that is characterised by figure-8 patterns of movement (Lopez-Garcia et al., 2008). This increased motility is necessary to ensure sufficient progress though the female reproductive tract and to ensure increased probability of sperm and egg interaction and subsequent fertilisation (Alasmari et al., 2013). There are a number of studies that indicate that hyperactivation is mediated by Ca$^{2+}$ signalling pathways (Carlson et al., 2003). It has been hypothesized that the asymmetric bending may be due to calcium binding directly to a subset of dynein arms (Lindemann and Kanous, 1997) or due to calcium binding to calmodulin receptors (Suarez, 2008).

Hyperactive motility may be sufficient to detach a sperm from the oviduct epithelium (Curtis et al., 2012). Although the specific role this plays during this complex process remains unclear, it is thought that hyperactivity enables sperm to escape mucosal folds in the oviduct and break bonds between the sperm head and the oviductal epithelium (Simons et al., 2014). In support of this, mouse sperm deficient in CatSper calcium channels that cannot hyperactivate are unable to detach from the oviduct (Ho et al., 2009). Furthermore, it is also thought that once sperm are released, hyperactive motility helps to promote the penetration of the complex, viscoelastic matrix surrounding the oocyte - the zona pellucida (Demott and Suarez, 1992).
Presently, to the best of the author’s knowledge, outside of our group, there have been no studies carried out in relation to BBD126 and BBD126 haplotype variation in cattle. A haplotype is a set of single-nucleotide polymorphisms (SNPs) on one chromosome that tend to be inherited together. The growing body of evidence linking β-defensins - specifically Defb126 - to sperm maturation, mucus penetration and sperm binding in rodents and primates leads us to characterise the effect of BBD126 and BBD126 haplotype on bull sperm motility and the binding of bull sperm to oviductal epithelia. Increased understanding of the role of BBD126 and BBD126 haplotypes in bull sperm motility and bull sperm binding holds significant opportunity for improved bovine fertility. It holds promise as a genetic test for bull fertility which would be useful especially for young genomically selected bulls.

The objectives of this thesis were to:

1. Optimise bovine oviductal epithelial cell (BOEC) monolayer and explant *in vitro* assays for the assessment of bull sperm binding.

2. Assess the role of BBD126 haplotype on the ability of bull sperm to bind to oviductal epithelium *in vitro*.

3. Assess the role of BBD126 haplotypes on sperm motility and kinematic parameters assessed using computer aided sperm analysis (CASA).

4. Assess the role of Recombinant BBD126 (rBBD126) on the ability of bull sperm to bind to BOEC *in vitro*. 
Chapter 2: Materials & Methods
Chapter 2: Materials & Methods

2.1 Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (Co Wicklow, Ireland) unless stated otherwise.

2.2 Experiment 1: Optimisation of BOEC Monolayer Binding Assay

(a) Effect of Cell Culture Support Material on BOEC Monolayer Confluence (%)

The aim of this experiment was to determine the most suitable support material for optimum BOEC monolayer growth. Material types assessed were (i) Glass cover slips, (ii) Glass cover slips with a fibronectin coating, (iii) Glass cover slips with a collagen coating and (iv) Millicell EZ slides (Merck Millipore, Cork, Ireland). These were used to determine the most suitable material for optimum BOEC monolayer confluence (%) over time. All cover slip growth materials were used with 24 well tissue culture plates (Sarstedt, Wexford, Ireland). Five replicates were completed.

(b) Effect of BOEC Seeding Rate on Confluence (%)

The aim of this experiment was to determine the most suitable BOEC seeding rate for optimum BOEC monolayer growth. Various BOEC seeding rates (1 x 10^6, 2 x 10^6, 3 x 10^6 and 4 x 10^6 cells per mL) were used to determine the most suitable seeding rate for optimum BOEC monolayer confluence (%) over time. Three replicates were completed.

BOEC Monolayer Binding Assay

From the preliminary data collected in experiment 1a and 1b, the following BOEC monolayer assay was used for all monolayer experiments. Non pregnant heifer reproductive tracts (n=3) were collected on site at a commercial abattoir immediately post-mortem and transported to the laboratory within 1h in sterile phosphate buffered saline (PBS)
supplemented with gentamicin (0.25 mg/mL) at 4°C. Reproductive tracts from heifers at various cycle stages were used, as stage has been shown to have no effect on sperm binding (Lefebvre et al., 1997, Gwathmey et al., 2003). At the laboratory, oviducts were trimmed free of connective tissue and ligated using single use sterile umbilical cord clamps (Dalhausen). Oviducts were sterilised in 70% ethanol for 30 sec and washed twice with PBS supplemented with Gentamicin (0.25 mg/mL).

Under laminar flow, the ligature was removed and the isthmic portion of the oviduct was dissected free of connective tissue. BOEC from each animal were recovered mechanically by squeezing each isthmus with a previously sterilised glass slide into separate petri P90 dishes (Sarstedt, Wexford, Ireland). BOEC were washed in 10 mL of PBS containing Gentamicin (0.25 mg/mL) at 1000 g for 10 min. Post centrifugation, the supernatant was removed, pellet diluted in fresh PBS containing Gentamicin (0.25 mg/mL) and re-centrifuged. Following this, previously prepared stock M199 culture medium supplemented with gentamicin (2.5 mg/mL) and fetal bovine serum (10%) was added to the BOEC of each reproductive tract. Cells were mechanically isolated using a 1000 uL pipette tip (aspirated and expelled 10 times) and by using a 1 mL syringe and 25G needle (aspirated and expelled 10 times).

BOEC concentration was assessed using a haemocytometer (Neubauer), adjusted to 1 x 10^6 cells per mL using warmed stock medium solution and seeded onto EZ slides. Cells were deemed viable for cell culture by a single evaluator by the observation of numerous beating cilia. Optimum seeding concentration and culture material was determined from experiments 1a and 1b. BOEC were incubated at 37°C and 5% CO₂ for six - seven days until fully confluent monolayers were formed. Every 48 h culture medium was replenished with stock culture medium and confluence (%) was assessed until fully confluent monolayers were formed (Figure 14 and 15).
To assess sperm binding frozen-thawed sperm samples were stained with 1% Hoechst 33342 (Al Naib et al., 2011), adjusted to 5 million per mL using warmed stock culture medium solution and added to the confluent monolayers. Co-cultures were incubated at 37°C and 5% CO₂ for 30 min. Loosely bound sperm were gently washed away with fresh medium and cultures were fixed with 2.5% glutaraldehyde. Sperm were observed under half-light and half fluorescence at 400X (BX60; Olympus, Centre Valley, PA, USA). The number of bound sperm was assessed by counting the number of bound sperm in ten random fields of view for each well. The evaluator was blinded to treatment for all sperm binding assessments. A colleague, who was independent of the study, numbered each treatment at random and recorded this sequence. This sequence was only shared with the evaluator post evaluation and data collation.

Figure 14: Confluency (40%) of monolayers on Day two of culture
2.3 Experiment 2: Optimisation of BOEC Explant Binding Assay

(a) Effect of Sperm Concentration on Sperm Binding Density to BOEC Explants

The aim of this experiment was to determine the optimum sperm concentration for BOEC explant binding. Various sperm concentrations (1 x 10^6, 2.5 x 10^6, and 5 x 10^6 per mL) were used to determine the most suitable concentration for optimum sperm binding density to explants. Five replicates were completed.

(b) Effect of Stage of Oestrous Cycle on Sperm Binding Density to BOEC Explants

The aim of this experiment was to determine the effect of oestrous cycle stage on the ability of sperm to bind BOEC explants. Heifer reproductive tracts (n=4) were collected, one from each stage of the oestrous cycle (1 - Oestrus, 2 - Metoestrus, 3 - Dioestrus, 4 - Prooestrus). Stage of cycle was estimated using morphological assessment of the ovaries (Ireland et al., 1979). BOEC from the tracts of the various stages of the oestrous cycle were recovered and

Figure 15: Confluency (90%) of monolayers on Day seven of culture
assessed for sperm binding density (5 x 10⁶ initial sperm concentration) to explants. Five replicates were completed.

**BOEC Explant Binding Assay**

From the preliminary experiments 2a and 2b, the following BOEC assay was used for all explant experiments. Heifer reproductive tracts (n=3) were collected and the oviducts were prepared in laboratory using the same dissection method as the monolayer assay. Under laminar flow, BOEC were isolated from each animal using the previously established mechanical isolation technique. BOEC were recollected and washed in 1 mL of PBS containing Gentamicin (0.25 mg/mL) at 200 g for 1 min. Post centrifugation, the supernatant was removed and stock M199 culture medium supplemented with gentamicin (2.5 mg/mL) and fetal bovine serum (10%); (1 mL) was added. BOEC were incubated at 37°C in 5% CO₂ for 1 h to allow formation of everted vesicles with apical ciliated surfaces oriented outwardly as described by Ignotz et. al (2007).

Sperm samples were assessed for concentration using a haemocytometer, stained with Hoechst 33342 and adjusted to 5.7 x 10⁶ per mL using warmed stock medium solution. Stock medium was added (5 mL) to explants of each tract and centrifuged at 200 g for 5 min. Post-centrifugation, the supernatant was removed and explants (20 uL) from each tract were added to sperm aliquots (140 uL). Final sperm concentration was 5 x 10⁶ per mL as determined by preliminary assay optimisation data. After 30 min incubation at 37°C in 5% CO₂ loosely bound sperm were removed from explants by gently pipetting through two 75 uL droplets of stock medium solution on a warmed 24 well culture plate (37°C). A droplet of each treatment (10 μL) was placed on a pre-warmed slide and a pre-warmed cover slip was added. Explants were viewed using a microscope at 400X fitted with a heated stage at
37°C (BX60; Olympus, Centre Valley, PA, USA) under half-light and half-fluorescence (Figure 16).

**Figure 16:** Representative image of corpus sperm bound to a bovine oviduct epithelial cell explant (BOEC). The green line indicates the overall BOEC explant, blue lines indicate sperm bound to epithelial cells (both ciliated and non-ciliated), the orange line indicates a group of cilia on a ciliated epithelial cell, the yellow line indicates a non-ciliated epithelial cell and the purple lines indicate unbound sperm. Image recorded at 400X using a fluorescent microscope at half light and half fluorescence.

The number of sperm bound was recorded and relative surface area of each explant was determined using a micrometre which was inserted into the eye piece of the microscope. Ten explants of each treatment were assessed at random for sperm binding density. Sperm binding density was calculated by determining the number of sperm bound per 0.1 mm² of explant surface. All treatments were assessed for sperm binding density. The evaluator was blinded to treatment for all sperm binding assessments using the previously described method (Section 2.2).
2.4 Experiment 3: Assessment of the Effect of BBD126 Haplotype on Sperm Function

A haplotype is a set of single-nucleotide polymorphisms (SNPs) on one chromosome that tend to be inherited together. The BBD126 haplotype was found in bulls of high fertility only (See Section 2.3.1). The aim of this experiment was to assess the effect this haplotype on sperm function. This was done by assessing sperm from high fertility bulls with (H+ive; n=4) and without (H-ive; n=4) the haplotype as well low fertility bulls without the haplotype (L-ive; n=4) for (a) Post Thaw Motility and Kinematic Parameters, (b) Ability of Bull Sperm to Bind to BOEC (i) Monolayers (ii) Explants.

2.4.1 Fertility Association Study & Bull Selection

In Ireland it is a legal requirement to record the date of birth of all calves and record the dam and sire. This data is held in a central national database by The Irish Cattle Breeding Federation (ICBF). The ICBF calculated sire conception rate for 7000 sires used for AI in Ireland. Adjusted bull fertility phenotypes (based on a minimum of 1000 inseminations) for 7000 Irish AI bulls were used to identify bulls of high and low fertility from the ICBF database. The adjusted bull fertility model is calculated by adjusting for the calving rate for a given bull by factors such as cow genotype, cow lactation, herd, AI technician, semen type and semen price (Berry et al., 2011). The most divergent bulls (n=150) were selected for targeted sequencing of β-defensin genes. The extremes of high and low fertility were defined as greater than one standard deviation from the mean of the adjusted fertility model (Mean = 0). An association study was performed by Dr Emma Finlay (Teagasc, Grange, Co. Meath) to identify genetic regions significantly associated with male fertility using R package GenABEL (Aulchenko et al., 2007). Quality control was performed with the check marker function and SNPs were examined for association to fertility using breed and the number of matings performed as fixed effects. The most significantly associated SNPs were located in a haplotype consisting of 94 SNPs over 138kb, including the β-defensin 126 gene. This
haplotype was found in bulls of high fertility only. Using the data from this study, high fertility bulls with (H+ive; n=4) and without (H-ive; n=4) the haplotype as well low fertility bulls without the haplotype (L-ive; n=4) were selected from the ICBF database. It was hypothesised that while all high fertility bulls do not have this haplotype, those that do are always high fertility, possibly due to their ability to bind BOEC. Therefore it was necessary to include high fertility with and without the haplotype as well as low fertility bulls without the haplotype.

**Table 2:** Table of bulls selected from fertility association study and their adjusted animal model fertility values. The adjusted animal model fertility values are calculated by adjusting the calving rate for a given bull by factors such as cow genotype, cow lactation, herd, artificial insemination technician, semen type and semen price. Bulls selected form the study include high fertility bulls with the BBD126 haplotype (H+ive, n=4) and high (H-ive, n=4) and low fertility bulls (L-ive, n=4) without the haplotype. All adjusted fertility values based on a minimum of 1000 inseminations for each bull.

<table>
<thead>
<tr>
<th>Bull Number</th>
<th>Haplotype</th>
<th>Adjusted animal model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H+ive</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>H+ive</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>H+ive</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>H+ive</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>H-ive</td>
<td>0.06</td>
</tr>
<tr>
<td>6</td>
<td>H-ive</td>
<td>0.06</td>
</tr>
<tr>
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<tr>
<td>8</td>
<td>H-ive</td>
<td>0.05</td>
</tr>
<tr>
<td>9</td>
<td>L-ive</td>
<td>-0.05</td>
</tr>
<tr>
<td>10</td>
<td>L-ive</td>
<td>-0.06</td>
</tr>
<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>L-ive</td>
<td>-0.06</td>
</tr>
</tbody>
</table>
(a) Effect of BBD126 Haplotype on Sperm Motility and Kinematic Parameters

The aim of this experiment was to assess the effect of BBD126 haplotype on sperm motility and kinematic parameters. Using the bulls selected from the fertility association study (Section 2.3.1), the post thaw motility and kinematic parameters of high fertility bulls with (H+ive; n=4) and without (H-ive; n=4) the haplotype as well low fertility bulls without the haplotype (L-ive; n=4) were assessed using Computer Aided Sperm Analysis (CASA; Microptics, Barcelona, Spain). Semen straws were thawed in a water bath at 39°C for 30 sec. Semen from each bull was assessed for sperm concentration and diluted to 20 x 10^6 sperm per mL in warmed PBS. A droplet of diluted semen (10 μL) from each was placed on a pre-warmed slide and a pre-warmed cover slip was added. At least five randomly selected microscopic fields and a minimum of 1000 sperm were assessed using a negative phase-contrast microscope at 100X Olympus BX60 fitted with a heated stage at 37°C (Figure 17). The CASA derived motility and kinematic parameters assessed were motility (Mean velocity (VAP) about 10 μm/s), progressive motility (sperm which display a forward progressive linear movement), straight line velocity (VSL; μm/s; the straight line distance from beginning to end of trach divided by time taken), average path velocity (VAP; μm/s; the spatial averaged path that eliminated the wobble of the sperm head), curvilinear velocity (VCL; μm/s; total distance travelled by a sperm during the acquisition divided by the time taken), lateral head displacement (LHD; μm; deviation of the sperm head from the average path), linearity (LIN; %; VSL/VCL x 100), straightness (STR; %; VSL/VAP x 100), lateral amplitude (ALH; μm/s; maximum amplitude of lateral head displacement) and beat central frequency (BCF; Hz; beat frequency of centroids crossing the average trajectory). Three straws per bull were assessed, so in total, twelve straws per haplotype were assessed.
(b) Effect of BBD126 on the Ability of Sperm to Bind BOEC

(i) Effect of BBD126 Haplotype on Sperm Binding to BOEC Monolayers

The aim of this experiment was to assess the effect of BBD126 haplotype on the ability of sperm to bind BOEC monolayers. Using the same bulls from the fertility association study described previously, sperm samples from high fertility bulls with (H+ive; n=4) and without (H-ive; n=4) the haplotype as well low fertility bulls without (L-ive; n=4) were assessed using the monolayer assay (Section 2.2). Three replicates were completed over a three-day period. A replicate consisted of three heifer reproductive tracts with all sperm treatments performed in duplicate on each individual tract on each day. Two straws per bull were assessed. Eight straws per haplotype were assessed.

(ii) Effect of BBD126 Haplotype on Sperm Binding to BOEC Explants

The aim of this experiment was to assess the effect of BBD126 haplotype on the ability of sperm to bind BOEC explants. The monolayer assay method was initially selected to assess
the effect of BBD126 haplotype as it was thought the use of uniform confluent monolayers would increase reproducibility of the assay conditions. However, as a result of BOEC cell de-differentiation – the loss of beating cilia within 24 hours of culture, it was decided to repeat the work using the more physiological and reproducible explant assay protocol. Explants remain in suspension, not adhering to the culture dish and as a result, the cells remain in their natural coherence to each other and maintain specific cell-to-cell interactions and beating cilia (Walter, 1995). No mitotic divisions occur, meaning that dedifferentiation is prevented resulting in a more physiological assay (Walter, 1995). To assess the effect of BBD126 haplotype, sperm from the bulls of varying haplotype used previously were assessed in the explant binding assay. Three replicates were completed over a three-day period. A replicate consisted of three heifer reproductive tracts with all sperm treatments performed in duplicate on each individual tract on each day. In total, twelve straws per haplotype were assessed.

2.5 Experiment 4: Effect of Stage of Sperm Maturation on the Ability of Sperm to Bind BOEC Explants.

(a) A comparison of the ability ejaculated, cauda and corpus sperm to bind to BOEC explants

The aim of this experiment was to compare the ability of (i) ejaculated (ii) cauda and (iii) corpus sperm to bind BOEC explants. Heifer reproductive tracts (n=3) were collected and prepared as described previously. Freshly ejaculated semen straws from different Holstein bulls (n=3) were obtained from National Cattle Breeding Centre (NCBC) and transported to the laboratory by courier the morning after collection. To obtain epididymal sperm, both testes were recovered from mature bulls (n=3) post slaughter at a commercial abattoir and transported within 1 h to the laboratory at 4°C. To recover sperm from the cauda epididymis,
a small incision was made in the cauda and the lumen of the deferent duct was cannulated with a blunted 22G needle. Sperm cells were then gently flushed through the cauda with a 5 mL syringe loaded with PBS at 37 °C (Druart et al., 2009). Due to the small diameter of the epididymal tubule, flushing of corpus sperm was not possible. The corpus epididymis was isolated and minced with a scalpel blade in a dish of PBS at 37 °C. Sperm from the cauda and corpus epididymis from each bull were kept separate (Fernandez-Fuertes et al., 2016). Samples were assessed for concentration using a haemocytometer, stained with Hoechst 33342 and assessed for sperm binding density using the explant assay procedure (Section 2.3). Three replicates were completed over a three-day period. A replicate consisted of three heifer reproductive tracts with all sperm treatments performed in duplicate on each individual tract on each day.

**(b) A comparison of the ability of frozen thawed and freshly ejaculated sperm to bind to BOEC explants**

The aim of this experiment was to compare the ability of frozen-thawed and fresh sperm to bind to BOEC explants. Frozen-thawed straws (n =3) from different Holstein bulls (n =3) and fresh semen straws from different Holstein bulls were pooled separately. Samples were then assessed for sperm binding density using the explant binding assay (Section 2.3). Three replicates were completed over a three-day period. A replicate consisted of three heifer reproductive tracts with all sperm treatments performed in duplicate on each individual tract on each day.
2.6 Experiment 5: Assessment of the Effect of rBBD126 on the Ability of Corpus Sperm to Bind BOEC explants

(a) Effect of rBBD126 Concentration on the Ability of Corpus Sperm to Bind BOEC Explants in vitro

BBD126 is highly resistant to standard methods of dissociation and it was not possible to remove BBD126 from sperm once attached to the sperm surface (Fernandez-Fuertes et al., 2016). As a result, it was decided to use corpus sperm as BBD126 is not expressed in the corpus of the epididymis and is the only model in which to assess sperm without BBD126 (Narciandi et. al., 2016). The aim of this experiment was to assess the effect of rBBD126 concentration on the ability of corpus sperm to bind BOEC explants. BOEC explants and epididymal semen were collected and prepared using the previously described method. Caudal sperm and corpus sperm were assessed for concentration and stained with Hoechst 33342. The rBBD126 protein used was previously expressed in an E.coli host using a carrier protein (Narciandi et al., 2016). The following rBBD126 concentration treatments (i) Cauda (ii) Corpus (+0 μg/mL), (iii) Corpus (+10 μg/mL), (iv) Corpus (+30 μg/mL) and (v) Corpus (+90 μg/mL) were assessed. Caudal and corpus sperm treatments were diluted to 5.7 x10^6 per mL using warmed stock medium solution and assessed for sperm binding density using the BOEC explant protocol (Section 2.3). Five replicates were completed over a five-day period. A replicate consisted of three heifer reproductive tracts with all sperm treatments performed in duplicate on each individual tract on each day.

(b) Effect of rBBD126 and BBD126 Ab on the Ability of Corpus Sperm to Bind BOEC Explants in vitro

It was hypothesised that the incubation of rBBD126 with BBD126 Ab would inhibit the effect of rBBD126. The aim of this experiment was to assess the effect of rBBD126 and
BBD126 Ab on the ability of corpus sperm to bind to BOEC explants. BOEC explants and epididymal semen were collected and prepared using the previously described methods. Caudal sperm and corpus sperm were assessed for sperm concentration and stained with Hoechst 33342. Experiment 5(a) demonstrated that +10 μg/mL rBBD126 had the largest increase in binding ability. In macaques, +30 μg/mL BBD126 Ab was found to produce the optimum inhibitory effect (Tollner et al., 2008a, Tollner et al., 2008b). The custom monoclonal BBD126 Ab used was prepared previously (Narciandi et al., 2016). An IgG control was also included to demonstrate specificity of the antibody. IgG1 Isotype control, from murine myeloma, was used as a control as it does not react with any proteins other than anti-mouse whole serum. As a result, the following sperm treatments were assessed (i) Cauda, (ii) Corpus (+0 μg/mL rBBD126), (iii) Corpus (+10 μg/mL rBBD126), (iv) Corpus (+30 μg/mL BBD126 Ab and +10 μg/mL rBBD126), (v) Corpus (+30 μg/mL BBD126 Ab), (vi) Corpus (+7 μg/mL IgG Ab and +10 μg/mL rBBD126) and (vii) Corpus (+7 μg/mL IgG). All treatments were assessed for sperm binding density using the established explant technique (Section 2.3). Five replicates were completed over a five-day period. A replicate consisted of three heifer reproductive tracts with all sperm treatments performed in duplicate on each individual tract on each day.

2.7 Experiment 6: Effect of rBBD126 on Corpus Sperm Agglutination (%)

(a) Effect of Recombinant β-defensin 126 (rBBD126) Concentration on Corpus Sperm Agglutination (%)

It was observed in experiment 5 that rBBD126 reduced corpus sperm agglutination. The aim of this experiment was to assess the effect of rBBD126 concentration on corpus sperm agglutination (%). Testes were recovered from mature bulls in a commercial abattoir and transported to the laboratory at 4°C. Caudal sperm and corpus sperm were recovered,
assessed for concentration and diluted to $20 \times 10^6$ sperm per mL in PBS. Using corpus sperm, the following rBBD126 concentration treatments were assessed: (i) Cauda, (ii) Corpus (+0 μg/mL), (iii) Corpus (+10 μg/mL), (iv) Corpus (+30 μg/mL) and (v) Corpus (+90 μg/mL). The incidence of sperm agglutination were assessed using nigrosin–eosin staining (sperm: stain ratio of 1:1; 0.068 M water-soluble nigrosin, 0.014 M water-soluble eosin and 0.116 M sodium citrate). Nigrosin-eosin stain (30 μL) was added to eppendorfs of each sperm treatment (30 μL) and a droplet of each (10 μL) was smeared on to a glass slide and allowed to dry at room temperature. Spermatozoa were viewed under a phase contrast microscope (x400) by one evaluator blinded to treatment. Fifty events were assessed in each count and the average of two counts was recorded. An event was described as the occurrence of a single sperm cell or an agglutinated mass of spermatozoa. The number of spermatozoa per agglutinated mass was also recorded. Agglutination percentage was calculated as the number of spermatozoa per agglutinated mass divided by total number of spermatozoa counted 100 (Holden et al., 2016). Five replicates were carried out over a five day period.

(b) Effect of rBBD126 and BBD126 Ab on Corpus Sperm Agglutination (%)

The aim of this experiment was to assess the effect of rBBD126 and BBD126 Ab on corpus sperm agglutination (%). Testes were recovered from the abattoir and caudal sperm and corpus sperm were recovered as previously described and assessed for sperm concentration. The following sperm treatments were assessed (i) Cauda, (ii) Corpus (+0 μg/mL rBBD126), (iii) Corpus (+10 μg/mL rBBD126), (iv) Corpus (+30 μg/mL BBD126 Ab and +10 μg/mL rBBD126), (v) Corpus (+30 μg/mL BBD126 Ab), (vi) Corpus (+7 μg/mL IgG Ab and +10
μg/mL rBBD126) and (vii) Corpus (+7 μg/mL IgG Ab). Four replicates were completed.

Sperm agglutination (%) was assessed as previously described.

2.7 Statistical Analysis

Data were examined for normality of distribution, tested for homogeneity of variance, transformed where appropriate and analysed in the Statistical Package for the Social Sciences (SPSS software, version 22, IBM, Chicago, IL). Repeated measures (ANOVA) was used to analyse monolayer confluency (%) optimisation data. Univariate (ANOVA) was used to analyse all other data. Sperm agglutination (%) and sperm motility (%) data were transformed using a square root transformation. The transformed data were used to calculate the P values; however, the corresponding means and standard error of the non-transformed data are presented in the results. Post hoc tests were carried using the Tukey test, a P value <0.05 was considered statistically significant and results were reported as the mean ± the standard error of the mean (s.e.m.).
Chapter 3: Results
Chapter 3: Results

3.1 Experiment 1: Optimisation of BOEC Monolayer Binding Assay

(a) Effect of Cell Culture Support Material on BOEC Monolayer Confluence (%)

There was an effect of material type on confluence (%) of BOEC over time (P<0.05; Figure 18). Millicell Ez slides produced a higher BOEC confluence (%) over time than collagen coated glass (P<0.05). There was no difference in BOEC confluence (%) over time between Millicell Ez slides, fibronectin coated glass or glass (P>0.05). BOEC confluence (%) increased in all treatments over a seven day period (P<0.05). There was no treatment by day interaction (P>0.05).

![Figure 18: Confluence (%) of bovine oviductal epithelial cell monolayers cultured on various support material types over time. n = 5 replicates. ns represents non significance (P>0.05). (Vertical error bars represent s.e.m.)](image)
(b) Effect of BOEC Seeding Rate on Confluence (%)

There was no effect of initial seeding rate on BOEC confluence (%), (P>0.05; Figure 19). BOEC confluence (%) increased in all treatments over an eight day period (P<0.05). There was no treatment by day interaction (P>0.05).

Figure 19: Confluence (%) of bovine oviductal epithelial cell monolayers seeded on Millicell Ez slides at various initial cell seeding rates over time. n = 3 replicates. (Vertical error bars represent s.e.m.)
3.2 Experiment 2: Optimisation of BOEC Explant Binding Assay

(a) Effect of Sperm Concentration on Sperm Binding Density to BOEC Explants

There was no effect of sperm concentration on sperm binding density to BOEC explants (P>0.05; Figure 20). Sperm binding density was 5.9 (±1.88), 7.6 (±2.97) and 13.6 (±1.15) per 0.1mm$^2$ of explant for sperm concentrations of $1 \times 10^6$/mL, $2.5 \times 10^6$/mL and $5 \times 10^6$/mL respectively.

**Figure 20:** Sperm binding density per 0.1mm$^2$ of explant following addition of frozen thawed sperm at various concentrations to bovine oviductal epithelial cell explants. n = 3 replicates. (Vertical error bars represent s.e.m.)
(b) Effect of Stage of Oestrous Cycle on Sperm Binding Density to BOEC Explants

There was no effect of stage of oestrous cycle on the ability of frozen thawed sperm to bind BOEC explants (P>0.05; Figure 21). Sperm binding density was 13.9 (±1.50), 15.0 (±1.00), 15.8 (±1.81) and 14.2 (±1.28) per 0.1mm$^2$ of explant for stages 1, 2, 3, and 4 of the oestrous cycle respectively.

**Figure 21:** Sperm binding density of sperm per 0.1mm$^2$ of explant to bovine oviductal epithelial cell explants in various stages of oestrous (1 - Oestrus, 2 - Metoestrus, 3 - Dioestrus, 4 - Prooestrus). n = 5 replicates. (Vertical error bars represent s.e.m.)
3.3 Experiment 3: Assessment of the Effect of BBD126 Haplotype on Sperm Function

(a) Effect of BBD126 Haplotype on Sperm Motility and Kinematic Parameters

There was no effect of BBD126 haplotype on post thaw sperm motility (P>0.05; Figure 22). Motility (%) was 48.5 (±3.60), 49.5 (±5.83) and 38.0 (±3.01) for sperm from H+ive, H-ive, and L-ive bulls respectively.

Figure 22: Post thaw motility (%) from bulls of varying haplotype (H+ive, n = 4; H-ive, n = 4; L-ive, n= 4) were assessed using CASA analysis. n = 3 replicates. Twelve straws per haplotype were assessed. (Vertical error bars represent s.e.m.)
There was no effect of BBD126 haplotype on progressive motility (%) 27.4 (±4.16), 25.8 (±5.74) and 23.72 (±2.89) for sperm from H+ive, H-ive, and L-ive bulls respectively (P>0.05; Figure 23). There was no effect of haplotype on any of the other kinematic parameters including VSL, VAP, VCL, LHD, LIN, STR, ALH and BCF (P>0.05).

**Figure 23:** Progressive motility (%) of sperm from bulls of varying haplotype (H +ive, n = 4; H-ive, n = 4; L-ive, n= 4) were assessed post thaw using CASA analysis. n = 3 replicates. Twelve straws per haplotype were assessed. (Vertical error bars represent s.e.m.)
(b) Effect of BBD126 Haplotype on Sperm Binding to BOEC

(i) Effect of BBD126 Haplotype on Sperm Binding to BOEC Monolayers

There was no effect of BBD126 haplotype on the number of sperm bound to confluent BOEC monolayers (P>0.05; Figure 24). No. of sperm bound (per ten fields of view) was found to be 40.3 (±5.89), 35.9 (±4.93) and 35.5 (±3.67) for sperm from H +ive, H-ive, and L-ive bulls, respectively.

Figure 24: No. of sperm bound (per ten fields of view) from bulls of varying haplotype (H +ive, n = 4; H-ive, n = 4; L-ive, n= 4) to confluent bovine oviductal epithelial cell monolayers. n = 2 replicates. Eight straws per haplotype were assessed. (Vertical error bars represent s.e.m.)
(ii) Effect of BBD126 Haplotype on Sperm Binding to BOEC Explants

There was an effect of BBD126 haplotype on sperm binding density to BOEC explants (P<0.05; Figure 25). Sperm from H+ive bulls bound to BOEC explants at a greater density than H-ive and L-ive bulls. Sperm binding density was 15.5 (±1.11), 12.3 (±0.91), 12.0 (±0.83) per 0.1mm$^2$ of explant for sperm from H+ive, H-ive, and L-ive bulls, respectively. There was an effect of bull on binding to BOEC explants (P<0.05; Figure 26). Most notably, however, there was no bull x haplotype interaction (P>0.05; Figure 26).

![Figure 25: Binding density of sperm from different haplotypes (H+ive, n = 4; H-ive, n = 4; L-ive, n= 4) to bovine oviductal epithelial cell explants. Binding density is expressed as the number of bound sperm per 0.1mm$^2$ of explant. $^{ab}$Different superscripts between treatment groups differ significantly (P<0.05). n = 3 replicates. Twelve straws per haplotype were assessed. (Vertical error bars represent s.e.m.)](image-url)
Figure 26: Binding density of sperm from individual bulls of different haplotypes (H+ive, n = 4; H-ive, n = 4; L-ive, n = 4) to bovine oviductal epithelial cell explants. Binding density is expressed as the number of bound sperm per 0.1mm$^2$ of explant. n=3 replicates. Three straws per bull were assessed. (Vertical error bars represent s.e.m.)
3.4 Experiment 4: Effect of Stage of Sperm Maturation on the Ability of Sperm to Bind BOEC Explants

(a) *A comparison of the ability of ejaculated, cauda and corpus sperm to bind to BOEC explants*

There was an effect of sperm maturity stage on sperm binding density to BOEC explants (P<0.05; Figure 27). Fresh sperm was had greater sperm binding density to BOEC explants in comparison to caudal and corpus sperm (P<0.05). There was no difference in sperm binding density between caudal and corpus sperm (P>0.05). Sperm binding density was 23.2 (±2.97), 15.2 (±2.88) and 13.2 (±1.56) per 0.1mm$^2$ of explant for ejaculated sperm, caudal sperm and corpus sperm, respectively (Figure 27).

![Figure 27: Binding density of ejaculated, cauda and corpus sperm to bovine oviduct epithelial cell explants. Binding density is expressed as the number of bound sperm per 0.1mm$^2$ of explant. (Vertical error bars represent s.e.m.) $^{ab}$Different superscripts between treatment groups differ significantly (P<0.05). n = 3 replicates.](image)
(b) A comparison of the ability of frozen thawed and freshly ejaculated sperm to bind to BOEC explants

There was no difference in sperm binding density between frozen thawed sperm and freshly ejaculated sperm (P>0.05, Figure 28). Sperm binding density was 17.4 (±2.13) and 23.2 (±2.97) per 0.1mm² of explant for frozen-thawed and fresh sperm, respectively.

**Figure 28:** Binding density of frozen ejaculated sperm and fresh ejaculated sperm to bovine oviduct epithelial cell explants. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. (Vertical error bars represent s.e.m.) n = 3 replicates. (Vertical error bars represent s.e.m.)
3.5 Experiment 5: To Assess the Effect of rBBD126 on the Ability of Corpus Sperm to Bind BOEC explants

(a) Effect of rBBD126 Concentration on the Ability of Corpus Sperm to Bind BOEC Explants in vitro

There was an effect of rBBD126 concentration on corpus sperm binding density to BOEC explants (P<0.05; Figure 29). Corpus sperm (+10 µg/mL rBBD126) had a greater sperm binding density to BOEC explants than corpus sperm (+0 µg/mL rBBD126, P<0.05). There was no difference in sperm binding density between the other treatments (P>0.05).

Figure 29: Binding density of cauda and corpus sperm incubated with various concentrations of recombinant BBD126 (rBBD126) to bovine oviduct epithelial cell explants. Corpus sperm without rBBD126 was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. (Vertical error bars represent s.e.m.)abDifferent superscripts between treatment groups differ significantly (P<0.05). n = 5 replicates.
(b) Effect of rBBD126 and BBD126 Ab on the Ability of Corpus Sperm to Bind BOEC Explants in vitro

There was an effect of rBBD126 (+10 µg/mL) and BBD126 antibody (+30 µg/mL) on corpus sperm binding density to BOEC explants; (P<0.05; Figure 30). Corpus sperm (+10 µg/mL rBBD126) had a greater sperm binding density to BOEC explants than corpus sperm (+0 µg/mL rBBD126, P<0.05). Furthermore, the presence of BBD126 antibody (+30 µg/mL) was found to inhibit the increase in sperm binding density associated with rBBD126 (+10 µg/mL, P<0.05). The presence of control protein IgG (+7 µg/mL) did not inhibit the stimulatory effect of rBBD126 (+10 µg/mL, P>0.05).

Figure 30: Binding density of cauda and corpus (Co) sperm to bovine oviduct epithelial cell explants following incubation with and without recombinant BBD126, BBD126 antibody and a control IgG mouse antibody. Corpus sperm without rBBD126 was used as a control. Binding density is expressed as the number of bound sperm per 0.1mm² of explant. (Vertical error bars represent s.e.m.) a,b,c,dTreatment groups with different superscripts differ significantly (P<0.05). n = 5 replicates.
3.6 Experiment 6: Effect of rBBD126 on Corpus Sperm Agglutination (%)

(a) Effect of Recombinant β-defensin 126 (rBBD126) Concentration on Corpus Sperm Agglutination (%)

There was an effect of rBBD126 concentration on corpus sperm agglutination (%) (Figure 31, P<0.05). Corpus sperm (+0 µg/mL rBBD126) exhibited higher sperm agglutination (%) than caudal and corpus sperm (+90 µg/mL rBBD126, P<0.05).

Figure 31: Sperm agglutination of cauda and corpus sperm following incubation with various concentrations of recombinant BBD126. (Vertical error bars represent s.e.m.) "ab"Different superscripts between treatment groups differ significantly (P<0.05). n = 5 replicates.
(b) Effect of rBBD126 and BBD126 Ab on Corpus Sperm Agglutination (%)

Sperm agglutination (%) was found to be 3.8 (±2.56), 14.8 (±4.21), 5.1 (±3.31), 15.3 (±3.90), 5.5 (±0.96), 7.3 (±2.14), 6.75 (±3.12) for caudal sperm, and corpus sperm incubated with (+0 µg/mL rBBD126), (+10 µg/mL rBBD126), (+30 µg/mL BBD126 antibody, +10 µg/mL rBBD126), (+30 µg/mL BBD126 antibody), (+7 µg/mL IgG) and (+7 µg/mL IgG & +10 µg/mL rBBD126) respectively. Vertical error bars represent s.e.m. There was an approaching significant effect of rBBD126 (+10 µg/mL) and BBD126 antibody (+30 µg/mL) on corpus sperm agglutination (%). (P = 0.07; Figure 32). However, the addition IgG alone also reduced agglutination indicating that inhibition of agglutination is not specific to BBD126.

![Figure 32](image_url)

**Figure 32**: Sperm agglutination of cauda and corpus (Co) sperm following incubation with and without recombinant BBD126, BBD126 antibody and a control IgG mouse antibody. (Vertical error bars represent s.e.m.) n = 5 replicates.
Chapter 4: Discussion
Chapter 4: Discussion

During maturation, immature sperm migrate through the epididymis where they are bathed in region-specific epididymal fluid which leads to a sequential addition, deletion, or modification of sperm surface proteins. These modifications lead to the acquisition of properties vital for survival and performance in the female reproductive tract (Tollner et al., 2012). In primates, evidence is now accumulating that one of these protein additions, β-defensin 126, is preferentially expressed in the male reproductive tract and plays an important role in sperm motility, mucus penetration and sperm binding (Dorin and Barratt, 2014, Cornwall, 2014, Tollner et al., 2012, Tollner et al., 2008a). β-defensin 126 has been recently characterised on bull sperm and in the caudal epithelium of the epididymis (Fernandez-Fuertes et al., 2016, Narciandi et al., 2016). However, the role of BBD126 and BBD126 haplotypes in mediating sperm function has not previously been investigated in cattle.

To the best of the author’s knowledge, this is the first study to assess the functional role of BBD126 and BBD126 haplotypes in bull sperm binding to oviductal epithelia and bull sperm motility in vitro, thus providing a novel insight into the role of this protein in bovine reproduction. This study has shown that 1) BOEC explants were found to be more suitable for the assessment of sperm binding ability than BOEC monolayers; 2) BBD126 haplotype had no effect sperm motility or progressive motility; 3) Sperm from bulls with the BBD126 haplotype had an increased ability to bind BOEC; 4) Corpus epididymal sperm had the ability to bind to BOEC; 5) rBBD126 increases the ability of corpus sperm to bind to BOEC, an effect that is not inhibited by BBD126 Ab and 6) rBBD126 has a non-specific effect on sperm agglutination.
In the present study, a comparison of monolayer and explant BOEC culture techniques was performed. In agreement with the scientific literature (Walter, 1995, Rottmayer et al., 2006, Teijeiro et al., 2011), explant culture was found to be a more physiological assay in terms of maintaining beating cilia and specific cell-to-cell interactions. In addition, it was confirmed that, as stated in previous studies (Ignotz et al., 2007, Gwathmey et al., 2006, Suarez, 2002), stage of oestrous cycle had no effect on the ability of sperm to bind BOEC.

The present study has demonstrated that BBD126 haplotype has no effect on post thaw sperm motility, progressive sperm motility or any of the kinematic parameters analysed. However, bulls of high fertility with the BBD126 haplotype (H+ive) had an increased ability to bind BOEC explants in comparison to bulls of high and low fertility without the haplotype (H-ive). In a previous study in humans, genotypic variation in the Defb126 gene was found to have an effect on male fertility (Tollner et al., 2011). Men with the homozygous genotype for a Defb126 mutant allele were found to have a reduced ability to penetrate viscous HA gel, a surrogate for cervical mucus in vitro (Tollner et al., 2011). Most notably, like the bulls in this study, with respect to sperm motility these men appeared normal and resembled men who possess the Defb126 allele (Tollner et al., 2011). In bulls, the BBD126 haplotype has been shown to have no effect on ability to penetrate cervical mucus (Beatriz Fernandez-Fuertes, personal communication), however, as evidenced by this present study BBD126 appears to have a functional role in sperm binding in bovines similar to its role in the macaque (Tollner et al., 2008a).

This finding is particularly noteworthy as despite the fact that a number of studies have focused on single sperm traits such as sperm motility and sperm morphology (Stalhammar et al., 1994), individually, none of these traits were correlated significantly with in vivo fertility (De Pauw et al., 2002). It must be noted, by combining the results of the performance
of spermatozoa from a number of these single sperm trait assays, models were produced showing a high correlation with fertility (Gillan et al., 2008). However, a positive correlation has been reported between sperm binding density *in vitro* and non-return rate as an individual sperm quality check (De Pauw et al., 2002). In relation to this study, it is possible that due to their enhanced ability to bind BOEC, sperm from bulls with the haplotype (H+ive) remain fertile for longer and as a result have a wider window of opportunity for successful AI.

Sperm possess compensable traits such as motility for which male fertility can be improved by the insemination of additional sperm and non-compensable traits such as plasma membrane sperm binding for which fertility is not improved by increased sperm numbers (Flowers, 2013). The relationship between pregnancy rate (PR) and the number of sperm used in the inseminate reaches a plateau beyond which additional sperm cause no increase in fertilisation (Flowers, 2013). With an increased emphasis on non-compensable traits such as binding in bull selection, numbers of sperm per AI could be lowered and costs reduced.

The present study has demonstrated that corpus and cauda epididymal sperm are able to bind to BOEC explants, although with an efficiency significantly less than freshly ejaculated sperm. Previous studies have shown that caudal epididymal sperm have the ability to bind BOEC *in vitro* (Gwathmey et al., 2003, Gualtieri et al., 2010); however, the present study is one of the only studies to assess the ability of corpus sperm to bind BOEC. Fresh and frozen semen produce comparable pregnancy rates in the field (Bucher et al., 2009). In previous studies, sperm cryopreservation was found to significantly reduce the sperm binding ability of ejaculated sperm (Gualtieri et al., 2010), however, in this study there was no difference between fresh and frozen ejaculated sperm in terms of sperm binding ability.
Due to the difficulty in removing BBD126 from the surface of bull sperm (Fernandez-Fuertes et al., 2016), corpus epididymal sperm, which do not express the protein (Narciandi et al., 2016), were used as an alternative model to study the role of BBD126 protein in sperm binding. In this study, rBBD126 was found to have a significant stimulatory effect on the ability of corpus sperm to bind BOEC. This stimulatory effect was inhibited by the presence of BBD126 antibody but remained uninhibited by the presence of the control IgG antibody. Whilst rBBD126 increased corpus sperm binding, it did not increase binding to the levels exhibited by ejaculated sperm.

As a result, it can be stated that BBD126 has a role in bovine sperm binding; however, it is most likely as an additional mechanism to the BSP binding proteins mechanisms (Ignotz et al., 2007, Suarez, 2002, Lefebvre et al., 1997, Suarez, 2016). Cauda and corpus sperm epididymal sperm lack BSP proteins but retain the ability to bind BOEC; this indicates that other proteins i.e. BBD126 might be involved in this adhesion event (Manjunath et al., 1994, Gwathmey et al., 2006, Gualtieri et al., 2010). Currently, the identity of binding molecule(s) for BBD126 on BOECs is unknown (Tollner et al., 2008a), however, the expression pattern of BBD126 on the bull sperm head (Fernandez-Fuertes et al., 2016) appears to be consistent with the location and pattern of sperm attachment to BOEC.

Corpus sperm agglutination is well documented in a number of species including bovines (Dacheux et al., 1983, Briz et al., 1995). In this study, rBBD126 was found to reduce corpus sperm agglutination. Agglutination was reduced to levels comparable with caudal sperm, where BBD126 is expressed in vivo. It is possible that the increase in sperm binding observed after incubation with rBBD126 is due to the sperm being disengaged from one another and being allowed to swim freely rather than a direct effect on the sperm binding mechanism itself. This would be consistent with the observation of reduced agglutination.
and concurrent increased motility in a related study by our group (Fernandez-Fuertes et al., 2016). In this current study, in contrast to sperm binding, agglutination was found to be inversely proportional to increasing BBD126 concentration and both BBD126 antibody and control IgG failed to abrogate the effects of rBBD126. This indicates that whilst BBD126 has a role in reducing agglutination, this effect is not protein specific. Previous studies, including one in rhesus monkeys, have reported that glycoproteins that are present in the cauda epididymis also reduce agglutination (Srivastav et al., 2004).

In conclusion, this study has demonstrated that the host defense peptide BBD126 plays a role in sperm binding and reducing sperm agglutination. High fertility bulls with the BBD126 haplotype have an enhanced ability to bind BOEC explants in vitro in comparison to bulls of comparable field fertility and post thaw motility but without the BBD126 haplotype. Furthermore, the addition of rBBD126 was found to enhance the ability of corpus sperm to bind to BOEC and to reduce sperm agglutination. These findings indicate that BBD126 plays a role in bovine sperm binding and in the reduction of agglutination; however, the underlying molecular events regulating these biochemical processes remain unknown. Understanding these mechanisms is the first step towards a more complete understanding of the role of BBD126 and the identification of biomarkers for accurately predicting fertility. Further research on BBD126 and related β-defensins will help expand our understanding of sperm binding and infertility in the bovine sperm.
Appendix

1. Preparation of Medium

1.1 Culture Medium

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (Co Wicklow, Ireland). Sterile stock culture medium (50mL) was prepared daily consisting of M199 medium (42.5mL), fetal bovine serum (5mL) and gentamicin sulphate solution (2.5 mL; 50 mg/mL).

1.2 PBS

PBS (1X) was made up using reagents and masses from the table below. Reagents listed were dissolved in 800 mL of H2O, pH was adjusted to 7.4 with HCl and H2O added to make 1 L.

<table>
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<th>Reagent</th>
<th>Mass Required</th>
<th>Final Concentration</th>
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<td>8 g</td>
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<tr>
<td>KCl</td>
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<tr>
<td>Na₂HPO₄</td>
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<td>100 mM</td>
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</table>
Bibliography


'Deficient human beta-defensin 1 underlies male infertility associated with poor sperm motility and genital tract infection', Sci Transl Med, 6(249), pp. 249ra108.


